Sensitive Detection of Viable *Listeria monocytogenes* by Reverse Transcription-PCR

Detection of pathogens in contaminated food products by PCR can result in false-positive data due to the amplification of DNA from nonviable cells. A new method based on reverse transcription-PCR (RT-PCR) amplification of mRNA for the specific detection of viable *Listeria monocytogenes* was developed. The expression of three *L. monocytogenes* genes, *iap*, *hly*, and *prfA*, was examined to determine a suitable target for amplification of RT-PCR. Total RNA from *L. monocytogenes* was isolated, and following DNase treatment, the RNA was amplified by both RT-PCR and PCR with primers specific for the three genes. Amplicon detection was accomplished by Southern hybridization to digoxigenin-labeled gene probes. The levels of expression of these three genes differed markedly, and the results indicated that the *iap* gene would provide a good target for development of a specific method for detection of viable *L. monocytogenes* based on RT-PCR amplification. After a 1-h enrichment, the 371-bp *iap*-specific product was detected with a sensitivity of ca. 10 to 15 CFU/ml from pure culture. Detection of the 713-bp *hly*-specific amplicon was ca. 4,000 times less sensitive after 1 h, whereas detection of the 508-bp *prfA* product showed the lowest level of sensitivity, with detection not observed until after a 5-h enrichment period. The amplification of the *iap* mRNA was specific for *L. monocytogenes*. Overall, the assay could be completed in ca. 54 h. The use of RT-PCR amplification for the detection of viable *L. monocytogenes* was validated in artificially contaminated cooked ground beef. Following a 2-h enrichment incubation, the *iap*-specific amplification product could be detected in a cooked meat sample that was originally inoculated with ca. 3 CFU/g. These results support the usefulness of RT-PCR amplification of mRNA as a sensitive method for the specific detection of viable *L. monocytogenes* and indicate that this method may prove useful in the detection of this pathogen in ready-to-eat, refrigerated meat products.

Listeria monocytogenes is a gram-positive intracellular organism causing severe infections that primarily affect pregnant women, newborns, and immunocompromised individuals (43). While *Listeria* spp. are ubiquitous in nature, only *L. monocytogenes* is pathogenic to humans. In recent years, a number of outbreaks of food-borne illness involving a wide range of foods have been linked to *L. monocytogenes* (16). Elimination of this organism from foods is extremely difficult due to it widespread distribution and ability to grow at refrigeration temperature (4°C). The prevention of further outbreaks of listeriosis will require validation of pathogen interventions around critical control points in food processing. The development of sensitive and specific methods for the detection of *L. monocytogenes* will play a major role in accomplishing this goal.

Traditional testing methods for the detection of *L. monocytogenes* have relied almost exclusively on the use of specific microbiological media to isolate and enumerate viable bacterial cells from foods followed by a series of biochemical and serological tests for final confirmation (16, 35). Conventional culture-based methods are labor-intensive and time-consuming, in many instances requiring 5 to 10 days to complete. To overcome these limitations, a number of molecular biology-based techniques for the rapid detection of *L. monocytogenes* have been developed in recent years, including immunoassays, nucleic acid-based hybridization assays, and PCR-based methods (for reviews, see references 20, 22, and 44). While the immunogenic approach is rapid, nonspecific detection due to cross-reactivity may occur. Nucleic acid-based hybridization assays are rapid and have high specificity. However, at least 10^3 to 10^4 DNA targets are required to achieve a detectable hybridization signal. Similarly, PCR amplification techniques show a high degree of specificity and have the added advantage of extreme sensitivity (for a review, see reference 44). However, a disadvantage of conventional PCR techniques is that both viable and nonviable cells may be detected (1, 34). This is overcome in many cases by inclusion of an enrichment step to dilute out any nonviable cells that may be present (23, 38, 40). However, the use of enrichment techniques prior to PCR can prolong analysis times, eliminating much of PCR's high-sensitivity advantage. New types of culture media designed to reduce enrichment times (17, 39) and the use of immunomagnetic particles to concentrate bacteria (12, 18, 26) have been reported. Although these techniques have been somewhat successful at decreasing the time necessary for pathogen detection, they may not be able to unequivocally demonstrate whether the cells are alive or dead. Therefore, an alternative method for detection of bacterial cells that combines sensitivity and specificity with the ability to differentiate between viable and nonviable cells is needed.

The present study was undertaken to develop a sensitive method for the detection of viable *L. monocytogenes* cells based on amplification of mRNA by reverse transcription-PCR (RT-PCR) technology. The expression of three different *L. monocytogenes* genes (*iap*, *hly*, and *prfA*) was examined to determine a suitable target for RT-PCR amplification. The *iap* gene codes for p60, a major extracellular protein of *L. monocytogenes* that is thought to be associated with invasion of

nonprofessional phagocytic cells (30). The *hly* gene encodes the 58-kDa virulence factor listeriolysin O (36), whereas the *prfA* gene codes for a 27.1-kDa protein that has been shown to positively regulate the expression of several *Listeria* virulence factors (10, 31, 37). Detection methods based on PCR amplification of the *iap* (8, 13, 38), *hly* (3, 4, 11, 17, 21, 23, 25, 38, 40), and *prfA* (11) gene sequences have been reported. This report details a sensitive RT-PCR-based detection system for viable *L. monocytogenes* cells. By combining RT-PCR amplification of *iap* mRNA with a 1-h enrichment incubation and amplicon detection by Southern hybridization, sensitivity values for the assay were found to be ca. 10 to 15 CFU/ml for pure *L. monocytogenes* cultures, with the total time required for positive identification being ca. 54 h. Furthermore, when cooked ground meat was artificially contaminated with *L. monocytogenes* at a level corresponding to ca. 3 CFU/g, the *iap*-specific RT-PCR amplification product was detected following a 2-h enrichment incubation. These results indicate the usefulness of RT-PCR amplification of mRNA as a sensitive method for the detection of viable *L. monocytogenes* in ready-to-eat, refrigerated meat products.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. *L. monocytogenes* Scott A was used for the initial RT-PCR studies, while *L. monocytogenes* Brie-1, V37, and GVN5LG and *L. inocua*, *L. ivanovii*, and *L. welshimeri* were used for specificity testing of the RT-PCR assay. All *Listeria* spp. and strains were obtained from the USDA-ARS Eastern Regional Research Center culture collection. Stock cultures were maintained in brain heart infusion broth and stored at 4°C throughout the course of this study. Each strain was grown by inoculating 0.01 ml of the stock culture into 50 ml of tryptic soy broth (TSB) and incubating the culture overnight at 37°C. For the determination of cell numbers, the cultures were serially diluted in 0.01% (wt/vol) peptone in water and plated onto tryptic soy agar (TSA) with a spiral plater (Spiral Systems, Cincinnati, Ohio), and the plates were incubated overnight at 37°C. All media were obtained from Difco Laboratories (Detroit, Mich.).

Sample preparation and enrichment procedure. For experiments involving pure cultures, flasks containing 50 ml of TSB were inoculated with 5 ml of the appropriate dilution of a cell suspension of an *L. monocytogenes* overnight culture to obtain between 3 and 3×10^4 CFU/ml. The samples were incubated at 37°C, and 10-ml aliquots were harvested after 1, 3, 5, and 7 h for extraction of total *L. monocytogenes* RNA. For specificity testing of the RT-PCR assay, *Listeria* spp. were grown overnight at 37°C in TSB, and 1-ml aliquots (ca. 3×10^9 CFU/ml) were harvested after 16 h for RNA isolation. For studies in a food matrix, 91% lean ground sirloin was obtained from a local retail market. The meat was cooked by autoclaving at 121°C for 15 min. Individual 2-g portions were aseptically weighed into filter stomacher bags (Spiral Biotech, Bethesda, Md.) and inoculated with 2 ml of the appropriate dilution of *L. monocytogenes* to obtain between 0.3 and 30 CFU/g. Negative controls consisted of a 2-g meat sample inoculated with 2 ml of 0.1% (wt/vol) peptone in water. Each sample was diluted with 18 ml of filter-sterilized phosphate-buffered saline containing 0.1% Tween 80. The meat samples were homogenized for 2 min in a Stomacher Lab-Blender 400 (Tekmar Company, Cincinnati, Ohio). Seven and one-half milliliters of the meat homogenate was transferred to 67.5 ml of TSB and incubated at 37°C. Ten-milliliter aliquots were collected after 2, 6, and 8 h of incubation for the extraction of total bacterial RNA. To examine the ability of the RT-PCR assay to discriminate between viable and nonviable cells, 50 ml of TSB was inoculated with 1 ml of the appropriate dilution from an overnight *L. monocytogenes* culture to give a final concentration of 10^6 CFU/ml. One-milliliter aliquots were harvested for both total RNA and genomic DNA extraction. The cells were then heat killed by autoclaving for 15 min at 121°C. Following the heat treatment, the culture was placed at 37°C, and after 0, 2, 4, and 6 h of incubation, 1-ml aliquots were removed for both RNA and DNA extraction. For all experiments, the cells were collected by centrifugation for 10 min at $4{,}100 \times g$. The supernatant fluid was aspirated, and the cell pellets were immediately frozen in a dry ice-ethanol bath.

RNA and DNA isolations. Total RNA from *Listeria* spp. was isolated from frozen cell pellets by using a commercial FastPrep RNA isolation kit according to the manufacturer's protocol (Bio101/Savant, La Jolla, Calif.). The precipitated RNA was resuspended in 55 μl of diethyl pyrocarbonate-treated sterile water and stored at −70°C for further use. Total genomic DNA was extracted from the frozen cell pellets by using the G-NOME DNA isolation kit according to the manufacturer's protocol (Bio101/Savant). The precipitated DNA was resuspended in 50 μl of sterile distilled water and stored at −20°C for further use.

Synthetic oligonucleotide primers. Oligonucleotide primers used in this study are shown in Table 1. The ELMIAPF and ELMIAPR primers, used for amplification of an *iap*-specific sequence, were previously reported by Bubert et al. (8). All other primer sets were designed by using Oligo 5.0 primer analysis software (National Biosciences, Inc., Plymouth, Minn.). External gene primers for RT-PCR amplification of the specific mRNA species (i.e., LMIAP, LMHLY, and LMPRFA) are designated with an E prefix, whereas the internal primer pairs used for making the digoxigenin (DIG)-labeled probes for Southern hybridization are designated with an I prefix. Forward 5′ primers and reverse 3′ primers are indicated with F and R suffixes, respectively. Oligonucleotides were synthesized at the Macromolecular Structure Analysis Facility of the University of Kentucky, Lexington.

TABLE 1. RT-PCR primers used for the detection of *L. monocytogenes* mRNA

Primers	Sequence (5′ to 3′)	Target gene	Size (position) of amplified product[a]
ELMIAPF	CAAACTGCTAACACAGCTACT	*iap*	371 (1178–1549)
ELMIAPR	GCACTTGAATTGCTCTTATTG		
ILMIAPF	ACAAGCAGCTCCAGTAGTTA	*iap*	119 (1240–1359)
ILMIAPR	CTGGTTTTGCAGCTTCTGTT		
ELMHLYF	TCCGCCTGCAAGTCCTAAGA	*hly*	713 (1620–2333)
ELMHLYR	GCGCTTGCAACTGCTCTTTA		
ILMHLYF	GCAATTTCGAGCCTAACCTA	*hly*	188 (1846–2034)
ILMHLYR	ACTGCGTTGTTAACGTTTGA		
ELMPRFAF	CGGGATAAAACCAAAACAATTT	*prfA*	508 (318–826)
ELMPRFAR	TGAGCTATGTGCGATGCCACTT		
ILMPRFAF	CAATGGGATCCACAAGAATA	*prfA*	186 (364–550)
ILMPRFAR	AGCCTGCTCGCTAATGACTT		

[a] Sizes are in base pairs; positions are in nucleotides.

RT-PCR and PCR amplifications. RT-PCR of *Listeria* RNA and PCR amplification of *Listeria* RNA or DNA was conducted with a GeneAmp PCR system 9600 thermal cycler and either the GeneAmp EZ r*Tth* (recombinant *Thermus thermophilus*) RNA PCR kit or the GeneAmp PCR reagent kit (Perkin-Elmer, Foster City, Calif.), respectively. For RNA amplification, 10 μl of an RNA sample was treated with 3 U of amplification-grade DNase I (Gibco BRL, Gaithersburg, Md.) in a total reaction volume of 20 μl for 20 min at 25°C. Following incubation, EDTA was added to a final concentration of 2.5 mM, and the sample was then incubated at 65°C for 10 min to inactivate the DNase. Half of the sample was then used in RT-PCR amplifications, and the other half was used for PCR amplification to serve as a control for DNA contamination. The RT-PCRs were performed in 50-μl volumes containing the following: 10 μl of DNase-treated RNA; 200 μM (each) dATP, dCTP, dGTP, and dTTP; 50 mM bicine (pH 8.2); 115 mM potassium acetate; 8% (wt/vol) glycerol; manganese acetate [$Mn(OAc)_2$; 1.5, 2.5, or 3.5 mM]; 0.5 μM each primer; and 5 U of recombinant *T. thermophilus* DNA polymerase. RT-PCR amplification conditions consisted of 1 cycle at 60°C for 35 min for RT of the RNA followed by a temperature cycling routine of denaturation at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min. After 40 cycles, a final extension at 72°C for 5 min was performed and the tubes were cooled to 4°C. For PCR amplification, reaction mixtures (total volume, 50 μl) contained the following: either 10 μl of DNase-treated RNA or 5 μl of genomic DNA; 200 μM (each) dATP, dCTP, dGTP, and dTTP; 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% (wt/vol) gelatin; 0.5 μM each primer; and 2.5 U of AmpliTaq DNA polymerase. The temperature cycling routine for denaturation, annealing, and extension was as described above. A 10-μl aliquot of each amplification reaction mixture was electrophoresed through a 1.5% agarose gel in Tris-acetate-EDTA buffer (TAE; 40 mM Tris-acetate [pH 8.0], 1 mM EDTA), and the gel was then stained with ethidium bromide.

DIG-labeled probe preparation. Three internal primer sets (ILMIAPF-ILMIAPR, ILMHLYF-ILMHLYR, and ILMPRFAF-ILMPRFAR) were employed for the generation of DIG-labeled DNA probes for use in detecting the amplified RT-PCR products. A 119-bp *iap*-specific probe, a 188-bp *hly*-specific probe, and a 186-bp *prfA*-specific probe were generated by incorporation of DIG-dUTP during PCR amplification according to the manufacturer's protocol (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Briefly, PCRs for the production of each gene-specific probe were performed in a final volume of 50 μl that contained the following: 200 μM (each) dATP, dCTP, and dGTP; 130 μM dTTP; 70 μM DIG–11-dUTP (Genius Systems; Boehringer Mannheim); 50 mM KCl; 1.5 mM $MgCl_2$; 10 mM Tris-HCl (pH 8.3); 0.01% (wt/vol) gelatin; 0.5 μM each primer; 2.5 U of AmpliTaq DNA polymerase; and 50 to 100 ng of

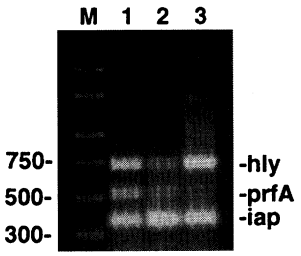

FIG. 1. Optimization of $Mn(OAc)_2$ concentration for RT-PCR amplification of *L. monocytogenes* Scott A RNA. Total RNA was extracted, treated with amplification-grade DNase I, and then amplified by RT-PCR using recombinant *T. thermophilus* DNA polymerase in reaction mixtures containing $Mn(OAc)_2$ concentrations of 1.5 mM (lane 1), 2.5 mM (lane 2), and 3.5 mM (lane 3) and all three sets of *L. monocytogenes*-specific primer pairs. The RT-PCR products were visualized by ethidium bromide staining of the agarose gel. Lane M, molecular size markers (in base pairs).

template genomic DNA. The conditions for temperature cycling for all PCR labeling procedures were 1 cycle at 95°C for 5 min followed by a 30-cycle temperature cycling routine of 95°C for 45 s, 60°C for 1 min, and 72°C for 2 min. Following amplification, a final extension at 72°C for 5 min was performed. An aliquot of each reaction product (5 μl) was electrophoresed through a 1.5% agarose gel in TAE buffer and then stained with ethidium bromide. The yield of DIG-labeled product was also estimated by spotting serial dilutions of the labeled probe along with serial dilutions of labeled control DNA (DIG-labeled pBR328 DNA [Boehringer Mannheim]) onto nylon membranes and comparing spot intensities following hybridization to anti-DIG-alkaline phosphatase and colorimetric detection according to the manufacturer's protocol (Boehringer Mannheim).

Southern hybridization and detection of amplification products. The electrophoresed amplification products were transferred to positively charged 0.45-μm-pore-size nylon membranes (Boehringer Mannheim) by using a Turboblotter transfer system according to the manufacturer's instructions (Schleicher and Schuell, Keene, N.H.). Following product transfer, the membranes were baked at 120°C for 30 min, placed in hybridization tubes, and prehybridized in buffer containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% Sarkosyl, 0.2% sodium dodecyl sulfate and 1.0% blocking reagent (Boehringer Mannheim) at 65°C for 4 h in a hybridization oven (model Mini 10; Hybaid Instruments, Holbrook, N.Y.). Hybridization was performed overnight at 65°C with fresh buffer solution containing an internal DIG-labeled gene-specific probe at a concentration of 2 ng/ml. Following washing of the membranes, the amplified products were detected colorimetrically with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate according to the manufacturer's protocol (Boehringer Mannheim).

RESULTS

Optimization of the RT-PCR assay. Experiments were conducted to determine the optimal salt concentration for RT-PCR amplification for each of the three *L. monocytogenes* primer pairs. Multiplex RT-PCRs were performed with the GeneAmp EZ r*Tth* RNA PCR kit (Perkin-Elmer) as recommended by the manufacturer, except that the $Mn(OAc)_2$ concentration was changed from 1.5 to 3.5 mM. The primers for amplification of *hly*, *prfA*, and *iap* were chosen such that the theoretical primer melting points for each primer pair were similar, allowing for a single annealing temperature of 60°C. When RT-PCR amplification mixtures contained 1.5 mM $Mn(OAc)_2$, three products of 713, 508, and 371 bp were observed, corresponding to the *hly*, *prfA*, and *iap* fragments, respectively (Fig. 1, lane 1). Increasing the $Mn(OAc)_2$ concentration to 3.5 mM resulted in an increase in amplification of the *hly* and *iap* products; however, amplification of the *prfA* product was inhibited (Fig. 1, compare lanes 1 and 3). Therefore, in subsequent experiments with either the *hly* or *iap* primer set, RT-PCRs were performed with 3.5 mM $Mn(OAc)_2$ whereas amplification of the *prfA* product was performed in reaction mixtures containing 1.5 mM $Mn(OAc)_2$.

Sensitivity of the RT-PCR. The suitability of each of the three *L. monocytogenes* genes to provide a sensitive target for detection of low levels of viable bacteria was examined by performing RT-PCRs on RNA isolated from broth cultures inoculated with serial dilutions of an overnight *L. monocytogenes* culture after various enrichment times. After 1 h of enrichment, the 371-bp *iap*-specific product was detected by Southern hybridization at levels corresponding to an initial inoculum of ca. 3 CFU/ml (Fig. 2, 1-h enrichment, lane 1). Enumeration on TSA plates indicated that after 1 h of incubation the culture contained ca. 10 to 15 CFU/ml. The *iap* product was the result of amplification of mRNA and not DNA, as evidenced by the lack of an amplification signal following PCR with AmpliTaq DNA polymerase (Fig. 2, lanes 6 to 10). When the RT-PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining after 1 h of enrichment, the 371-bp *iap* fragment was first detected in the sample initially inoculated at a level of ca. 3,000 CFU/ml (data not shown). Enumeration on TSA plates revealed that this sample contained ca. 5,000 CFU/ml, indicating that detection of the RT-PCR product by Southern hybridization was approximately 500 times more sensitive than detection by ethidium bromide staining. The *hly*-specific fragment was also detected by RT-PCR after a 1-h incubation period. The level of sensitivity for this message, however, was approximately 4,000-fold lower than that for the *iap* message (Fig. 3, 1-h enrichment, lane 5). The *hly* amplified product was detected only in cultures initially inoculated at the highest inoculum level of 3×10^4 CFU/ml. Enumeration of this culture on TSA after 1 h of enrichment indicated that it had a bacterial count of approximately 4×10^4 CFU/ml. Detection of the amplified *hly* message at each successive enrichment time was not observed until the culture reached a density of ca. 4×10^4 CFU/ml (Fig. 3, 3-h enrichment, lane 4; 5-h enrichment, lane 3; 7-h enrichment, lane 2). Detection of the *prfA* message by RT-PCR showed the lowest level of sensitivity among the three genes examined (Fig. 4). The 508-bp *prfA* product was first

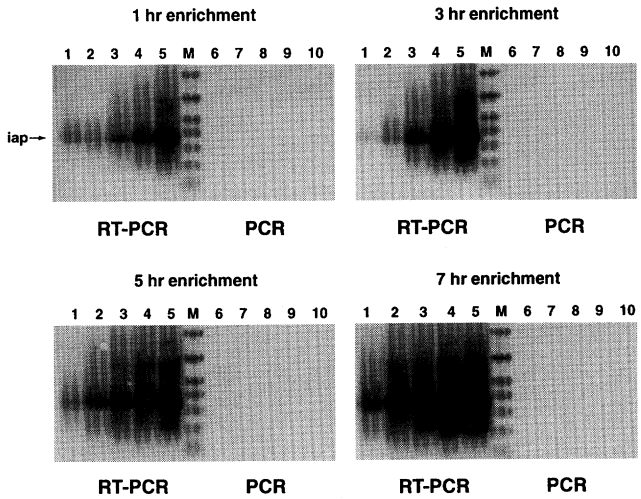

FIG. 2. Southern blot analysis of RT-PCR products of serially diluted *L. monocytogenes* Scott A mRNA amplified with *iap* gene-specific primers. Following enrichment for 1, 3, 5, or 7 h, total RNA was extracted, DNase treated, and amplified by RT-PCR (lanes 1 to 5) or PCR (lanes 6 to 10). The amplification products were colorimetrically detected following transfer to nylon membranes and hybridization with a DIG-labeled 119-bp internal *iap* probe. Lanes: 1 and 6, 10^{-9} dilution of overnight culture, 3-CFU/ml initial inoculum; 2 and 7, 10^{-8} dilution, 30-CFU/ml initial inoculum; 3 and 8, 10^{-7} dilution, 300-CFU/ml initial inoculum; 4 and 9, 10^{-6} dilution, 3,000-CFU/ml initial inoculum; 5 and 10, 10^{-5} dilution, 3×10^4-CFU/ml initial inoculum; M, DIG-labeled molecular size markers ranging from 1,000 to 100 bp (Boehringer Mannheim).

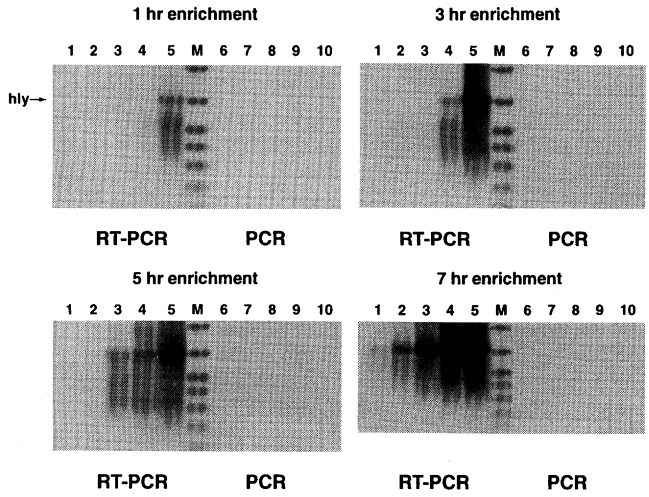

FIG. 3. Southern blot analysis of RT-PCR products of serially diluted *L. monocytogenes* Scott A mRNA amplified with *hly* gene-specific primers. Methods and lane designations are as described in the legend to Fig. 2, except that *hly* primers were used for RT-PCR amplification and product detection.

detected after the 5-h incubation period and then only in the culture originally inoculated at a level of 3×10^4 CFU/ml (Fig. 4, lane 5 of 5-h enrichment). After 5 h of incubation, the plate count for this culture was approximately 3×10^6 CFU/ml, and detection of the *prfA* message after 7 h of incubation was not observed except in those cultures with a density greater than 2.5×10^6 to 3×10^6 CFU/ml (Fig. 4, 7-h enrichment, lanes 3 to 5). Two bands were observed on the blot when the *prfA* primer set was used for amplification of *L. monocytogenes* mRNA. The *prfA* primers should generate a product of 508 bp. The two bands detected probably resulted from internal initiation of the 5′ primer during RT-PCR amplification since analysis of these primers with the Oligo 5.0 primer analysis software indicated a potential false-priming site approximately 75 bp inside the 5′ primer binding site (data not shown).

Specificity of the RT-PCR assay. The ELMIAPF-ELMIAPR primer pair (Table 1) has previously been shown to specifically

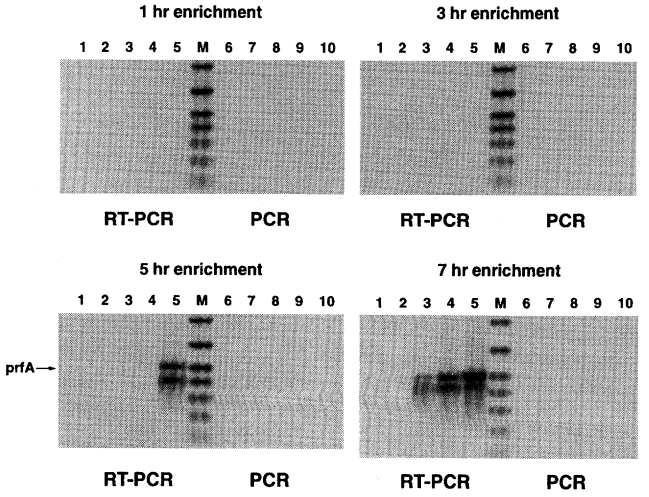

FIG. 4. Southern blot analysis of RT-PCR products of serially diluted *L. monocytogenes* Scott A mRNA amplified with *prfA* gene-specific primers. Methods and lane designations are as described in the legend to Fig. 2, except that *prfA* primers were used for RT-PCR amplification and product detection.

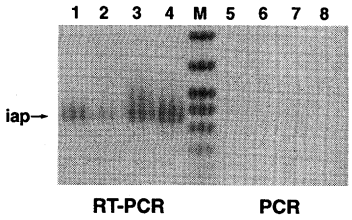

FIG. 5. Southern blot analysis of the RT-PCR-amplified *iap* products from different *L. monocytogenes* strains. Following a 1-h enrichment, total RNA was extracted from each strain, DNase treated, and amplified by RT-PCR (lanes 1 to 4) and PCR (lanes 5 to 8) with the *iap* primer set. Detection of the amplification products was performed by Southern analysis as described in Materials and Methods. Lanes: 1 and 5, *L. monocytogenes* Brie-1; 2 and 6, *L. monocytogenes* V37; 3 and 7, *L. monocytogenes* GVN5LG; 4 and 8, *L. monocytogenes* Scott A; M, DIG-labeled molecular size markers.

amplify a portion of the *iap* genes from numerous *L. monocytogenes* strains (8). Results of amplification of DNA isolated from *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, and *L. murray* by PCR with these primers were all negative (8). To extend these results to the present RT-PCR assay, we tested the specificity of the *iap* primer pair with four *L. monocytogenes* strains and three other *Listeria* spp. RT-PCR amplification of the 371-bp *iap*-specific fragment was detected with all four of the *L. monocytogenes* strains analyzed, and the sensitivity of the assay after a 1-h enrichment incubation was again ca. 10 to 15 CFU/ml for a pure broth culture (Fig. 5). However, when RNA was extracted from broth cultures of *L. innocua*, *L. ivanovii*, or *L. welshimeri* containing approximately 3×10^9 cells per ml and amplified by RT-PCR with the *iap* primer set, no detectable product was observed following Southern hybridization (data not shown). These results indicate that RT-PCR amplification of mRNA with the *iap* primers can be used for the specific identification of *L. monocytogenes*.

Ability of the RT-PCR assay to discriminate between viable and nonviable cells. The ability of the RT-PCR assay to distinguish between viable and nonviable *L. monocytogenes* cells was examined by performing RT-PCR as well as PCR assays with RNA and DNA isolated from both live and heat-killed cells. When *L. monocytogenes* mRNA and DNA were extracted from live cells and amplified by RT-PCR and PCR, respectively, the 371-bp *iap* product was observed as expected (Fig. 6, lanes L). Furthermore, the *iap* gene sequence was detected by conventional DNA-based PCR immediately following heat treatment of the cells and for at least 6 h after heating (Fig. 6, PCR panel, lanes 0, 2, 4, and 6 h). Enumeration of the culture on TSA plates revealed that autoclaving had rendered the cell culture nonviable, indicating that the bacterial DNA was quite

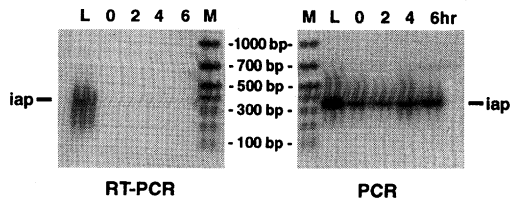

FIG. 6. Detection of *iap* mRNA and *iap* gene sequences in live and heat-killed *L. monocytogenes* cells by RT-PCR and PCR, respectively. Total RNA and genomic DNA were extracted from viable *L. monocytogenes* (lanes marked L) and from heat-killed cells (autoclaved at 121°C for 15 min) 0, 2, 4, and 6 h after heating. The RNA was DNase treated and amplified by RT-PCR (left panel), and the isolated DNA was amplified by conventional PCR (right panel). Detection of the amplification products was performed by Southern analysis as described in Materials and Methods. Lane M, DIG-labeled molecular size markers.

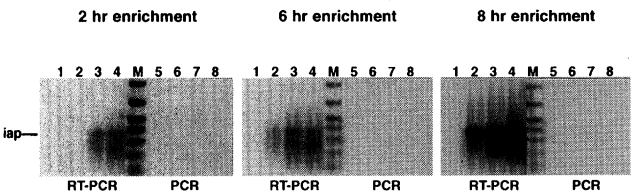

FIG. 7. Detection of *iap* mRNA in artificially contaminated cooked beef by RT-PCR. Cooked ground meat was inoculated with serial dilutions of *L. monocytogenes* cells, and the samples were incubated for 2, 6, or 8 h. Following enrichment, RNA was extracted, DNase treated, and amplified by RT-PCR (lanes 1 to 4) or PCR (lanes 5 to 8). The amplification products were colorimetrically detected following transfer to nylon membranes and hybridization with the DIG-labeled internal *iap* probe. Lanes: 1 and 5, uninoculated meat control; 2 and 6, 0.3-CFU/g initial inoculum; 3 and 7, 3-CFU/g initial inoculum; 4 and 8, 30-CFU/g initial inoculum; M, DIG-labeled molecular size markers.

stable and capable of being amplified for hours following the loss of viability. In contrast, *iap* mRNA from heat-killed cells could not be amplified by RT-PCR at any time following the heat treatment (Fig. 6, RT-PCR panel, lanes 0, 2, 4, and 6 h) due to the rapid degradation of the mRNA following the loss of cell viability. Thus, the detection of mRNA by RT-PCR amplification should provide a more sensitive indicator of cell viability than detection of gene sequences by DNA-based PCR amplification.

Validation of the RT-PCR detection assay with a food product. The ability to detect viable *L. monocytogenes* cells in a food product by RT-PCR amplification of *iap* mRNA was examined. Cooked beef samples (autoclaved at 121°C for 15 min) were artificially contaminated with serial dilutions of *L. monocytogenes* ranging from ca. 0.3 to 30 CFU/g, and following enrichment incubation the RNA was isolated and then amplified by RT-PCR. Following a 2-h enrichment incubation, the *iap*-specific product was detected in cooked meat samples that had been originally inoculated with ca. 3 CFU/g (Fig. 7, 2-h enrichment, lane 3). Enumeration on TSA plates was unable to give an accurate bacterial count for this sample, as the bacterial population was too low; however, following the 2-h enrichment incubation, an *iap*-specific RT-PCR product was also detected in the cooked meat sample initially inoculated with 30 CFU/g, and enumeration of this sample on TSA indicated that there was a bacterial population of ca. 100 CFU/ml of enrichment medium. Furthermore, after 6 h of enrichment incubation, the 371-bp *iap* product could be detected by Southern hybridization at levels corresponding to an initial inoculum level of <1 CFU/g. As was observed with pure broth cultures (Fig. 2), the *iap* product resulted from amplification of mRNA, not DNA, as evidenced by the lack of a signal when PCRs were performed (Fig. 7, lanes 5 to 8). Examination of Fig. 7 reveals that a second, lower-molecular-weight RT-PCR product was amplified in the cooked meat samples (2-h enrichment, lanes 3 and 4; 6-h enrichment, lanes 2 to 4). This second RT-PCR product was not due to amplification of contaminating DNA or RNA in the meat samples, as no RT-PCR or PCR product was amplified in uninoculated meat controls (Fig. 7, lanes 1 and 5). While we have not determined the exact cause of this doublet, the second product is a consequence of RT-PCR amplification of *L. monocytogenes* RNA isolated from the cooked meat samples. Elimination of this lower-molecular-weight product may require extensive optimization of the RT or PCR conditions with RNA samples purified from food matrices. Nevertheless, the presence of the doublet does not detract from the fact that low levels of *L. monocytogenes* mRNA can be detected in artificially contaminated cooked meat with the present RT-PCR parameters. Thus, the amplification of *iap* mRNA by RT-PCR may provide a means for sensitive and specific detection of viable *L. monocytogenes* cells in cooked meat products.

DISCUSSION

Traditional microbiological assays for the identification of *Listeria* species are time-consuming and often yield differing results (16). In recent years, more rapid methods for the detection of *Listeria* species, and in particular *L. monocytogenes*, have been developed. Many of these rapid methods are based on PCR amplification of specific *L. monocytogenes* gene sequences combined with ethidium bromide staining of agarose gels (11, 17, 23, 25, 33, 38), Southern (21, 46) or dot blot (4, 7) hybridization with labeled probes, or, more recently, with a fluorogenic probe incorporated into the PCR assay itself (TaqMan PCR) (3). Although these PCR-based detection methods have enhanced the sensitivity and specificity for rapid identification of *L. monocytogenes*, they can yield false-positive results due to the amplification of DNA present in nonviable cells (1, 34). It has been demonstrated that cells of *Escherichia coli* and *L. monocytogenes* that have been rendered nonviable, as determined by viable plate counts, by starvation, desiccation, or heat are still detectable by gene probe-PCR techniques, indicating the lack of a clear relationship between the loss of viability and detectability by PCR (34) (Fig. 6). In the present study, the *iap* gene sequence was amplified by conventional PCR 6 h after the cells had been killed by autoclaving. In addition, Baez et al. (1) observed false-positive PCR results when assessing the presence of *Clostridium perfringens* in beef following irradiation. Hence, under many circumstances which render bacteria nonviable, cellular DNA may persist for hours or even days, suggesting that detection of pathogens in contaminated foods by PCR may require additional evidence of cell viability before risk can be assessed (1, 34).

To overcome the potential for false-positive results in PCR-based assays, we have developed a method for the specific detection of *L. monocytogenes* based on RT-PCR amplification of mRNA. Most bacterial mRNAs have a very short half-life, on the order of 0.5 to 2 min, due to rapid degradation by endogenous RNases (27). Processes which render cells nonviable and hence disrupt cellular transcription will result in the rapid loss of cellular mRNA. An assay system based on detection of mRNA should provide a sensitive indicator of cell viability compared with methods that rely on the amplification of DNA or rRNA. Engstrand et al. (14) used an RT-PCR method for the detection of *Helicobacter* species; however, their method relied on the amplification of 16S rRNA, not mRNA. Like DNA, rRNA is extremely stable (27) and, therefore, may not be a suitable target for discriminating between viable and nonviable cells. We were unable to detect *iap* mRNA amplification by the RT-PCR assay following exposure of the cells to extreme heat (autoclaving at 121°C for 15 min), indicating the sensitivity of bacterial mRNA to rapid degradation as well as the tight association between cellular mRNA and cell viability. To our knowledge, this is the first report of a sensitive and specific method for the detection of viable *L. monocytogenes* based on RT-PCR amplification of mRNA.

A major limitation of RT-PCR-based detection systems is the difficulty of rapidly isolating undegraded mRNA from bacterial cultures due to its very short half-life. To address this problem, we evaluated the newly developed FastPrep RNA isolation system (Bio101/Savant) for the extraction of total RNA from bacterial cells. This system, which includes a FastPrep cell disruption instrument in conjunction with a FastRNA kit, was able to efficiently lyse cells and stabilize the RNA

before degradation occurred. The system was rapid, with total RNA extraction from cells accomplished within 1 h. We compared RNA isolation with the FastPrep system to isolation with TRIzol reagent (Gibco BRL). Although both systems were rapid and easy to use, the FastPrep system was more efficient at isolation of RNA from small numbers of cells (data not shown). The FastPrep system was found to rapidly yield mRNA suitable for RT-PCR amplification, requiring far less time and labor than conventional RNA isolation systems (41). However, some DNA contamination was present in the RNA samples. To avoid subsequent amplification of the DNA, a short DNase treatment step was incorporated prior to RT-PCR.

Successful detection of *L. monocytogenes* by RT-PCR of mRNA requires the selection of a suitable target gene for amplification. This target gene should possess several characteristics, including abundant transcript expression, expression throughout the growth cycle, and little or no transcriptional regulation. In the present study, we examined the amplification of three different *L. monocytogenes* mRNAs, *iap*, *hly*, and *prfA*, for suitability as targets for development of an RT-PCR detection system. DNA-based PCR detection methods using primers from these three genes have been developed (3, 4, 11, 13, 17, 21, 23, 25, 38, 40). In the present system, detection of the three mRNAs required a short enrichment incubation prior to total RNA extraction. This was necessary to increase the sensitivity of the detection assay. Confirmation of the RT-PCR products was accomplished by Southern hybridization with internal DIG-labeled probes from each of the three genes. Southern hybridization appeared to be at least 500 times more sensitive in amplicon detection than ethidium bromide staining of agarose gels. Comparison of the *iap*, *hly*, and *prfA* genes by this method indicated that the *iap* message was an ideal target for amplification by RT-PCR. Following a 1-h incubation step, we were able to detect the *iap*-specific product at a sensitivity of ca. 10 to 15 CFU/ml in pure broth cultures. Amplification of the *hly* message was approximately 4,000 times less sensitive, and amplification of *prfA* mRNA showed the lowest level of sensitivity of the three genes examined. The *iap* gene codes for p60, a major extracellular protein of *Listeria* species (9, 30). Wuenscher et al. (47) have demonstrated that the *iap* gene from *L. monocytogenes* is essential for cell viability and suggested that p60 is an essential housekeeping protein that is required during a late step in cell division. Furthermore, Köhler et al. (29) have shown that expression of the *iap* gene of *L. monocytogenes* is controlled at the posttranscriptional level. In mutants with reduced levels of p60, transcription of the *iap* gene appeared normal and wild-type levels of *iap* mRNA accumulated (29). These results indicate that the *iap* gene is constitutively expressed and, therefore, that *iap* mRNA should be present throughout the *L. monocytogenes* growth cycle. The present RT-PCR results with *iap* mRNA support this conclusion. In addition, the sensitivity observed when amplifying the *iap* mRNA indicates that this message is highly abundant within the cell. In contrast to the expression of the *iap* gene, the *hly* and *prfA* genes are not constitutively expressed (37) but are transcriptionally controlled. The *prfA* gene encodes a 27.1-kDa DNA-binding protein (19) that has been shown to positively regulate the expression of many of the *L. monocytogenes* virulence genes, including *hly* and *prfA* itself (10, 31, 37). Furthermore, the PrfA protein has also been shown to down regulate its own expression (19). Transcription of both *prfA* and *hly* is environmentally modulated and is subject to growth phase and thermal regulation (32, 37). Transcription of *hly* and *prfA* shows two peaks along the *Listeria* growth curve, one during early exponential growth and a second in early stationary phase (37). In addition, growth of listeria at temperatures less than 30°C inhibits transcription of both the *hly* and *prfA* genes (32). Based on these observations, the *hly* and *prfA* mRNAs would not be good candidates for the rapid detection of *L. monocytogenes* by RT-PCR. This observation is confirmed by the results of the present study. Both mRNAs appeared to be expressed at very low levels. We found that detection of the amplified *hly* product required ca. 4×10^4 CFU/ml whereas detection of the *prfA* amplicon required ca. 2.5×10^6 to 3×10^6 CFU/ml. In contrast, the *iap*-specific product was detected after a 1-h incubation from cultures containing ca. 10 to 15 CFU/ml.

The present results indicated that the *iap* primer set used for RT-PCR amplification was specific for *L. monocytogenes* and that the sensitivity of the assay was similar for all four strains tested. The *iap* gene is present in all *Listeria* species except *L. grayii* (28). No sequence homologous to an *iap* gene probe was detected in *E. coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Streptococcus pyogenes*, or *Bacillus subtilis* (28). The *iap* primer set used for RT-PCR in the present study and designed by Bubert et al. (8) for the specific detection of *L. monocytogenes* is derived from a region of the *iap* gene that is unique to *L. monocytogenes*. Based on the sensitivity and specificity observed with the *iap* primer set employed, it is concluded that the *iap* gene is the ideal target for sensitive and specific detection of *L. monocytogenes* by RT-PCR amplification of mRNA.

We have validated the use of the RT-PCR assay as a sensitive detection method for viable *L. monocytogenes* in artificially contaminated cooked meat. This organism is known to occur in both raw and cooked meats (6, 24) and is a pathogen of concern in cook-chill, ready-to-eat foods because of its ability to grow at refrigeration temperatures (45). Furthermore, there is sufficient evidence that *L. monocytogenes* can survive in ground meat following some thermal processing methods (5, 15, 42). In the present study, we chose to simulate a cooked meat product by autoclaving ground meat for 15 min at 121°C. Autoclaved meat may not be identical to most cooked meat products as far as the microbial population present; however, in our initial experiments we chose not to complicate the system with the presence of background microflora. Initially, these experiments were performed to ensure that the food matrix itself would not be inhibitory for the isolation of bacterial mRNA and its subsequent amplification by RT-PCR. Further studies must be conducted to determine what effect the presence of background flora might have on the RT-PCR assay we have developed. These studies will indicate whether a selective enrichment medium will be required for the isolation of *L. monocytogenes* in the presence of a natural microbial population. The results from the present study indicate that *L. monocytogenes* mRNA can be isolated from a food matrix artificially contaminated with very low levels of this organism. When cooked meat was contaminated with *L. monocytogenes* at an initial inoculum of ca. 3 CFU/g, the *iap*-specific RT-PCR product could be detected following a 2-h enrichment incubation. Furthermore, following a 6-h enrichment, we were able to detect *iap* mRNA by RT-PCR in a sample initially inoculated with <1 CFU/g, indicating that the food matrix did not inhibit bacterial RNA extraction or the RT-PCR assay itself. Although a short enrichment incubation (ca. 2 h) was necessary to increase the sensitivity of the RT-PCR assay, our results and those of others (1, 34) indicate that nonviable cells would be detected following this short enrichment period if DNA-based PCR detection systems were used. Thus, the present assay incorporating RT-PCR technology for the detection of bacterial mRNA would yield fewer false-positive results.

In conclusion, we have developed an RT-PCR assay for the sensitive detection of viable *L. monocytogenes*. This method is based on amplification of the *iap* mRNA by RT-PCR and combines a short enrichment incubation with total RNA extraction prior to amplification. Overall, the assay required ca. 54 to 55 h to complete. This is ca. 2 to 7 days shorter than traditional, culture-based testing methods for the identification of *L. monocytogenes*. We are currently examining alternative methods for amplicon detection in an effort to decrease the assay time further. The use of a chemiluminescence enzyme immunoassay for the detection of the RT-PCR product could reduce the assay time to less than 12 h while still providing a level of sensitivity comparable to that of Southern hybridization (2). Furthermore, experiments are under way to examine the effect of thermal injury on the isolation and detection of viable *L. monocytogenes* by RT-PCR in artificially as well as naturally contaminated food products.

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